

AXMI-008, A DELTA-ENDOTOXIN GENE AND METHODS FOR ITS USE

CROSS REFERENCE TO RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application Serial No. 60/448,797, filed February 20, 2003, the contents of which are herein incorporated by reference in their entirety.

FIELD OF THE INVENTION

This invention relates to the field of molecular biology. Provided are novel genes that encode pesticidal proteins. These proteins and the nucleic acid sequences that 5 encode them are useful in preparing pesticidal formulations and in the production of transgenic pest-resistant plants.

BACKGROUND OF THE INVENTION

Bacillus thuringiensis is a Gram-positive spore forming soil bacterium 10 characterized by its ability to produce crystalline inclusions that are specifically toxic to certain orders and species of insects, but are harmless to plants and other non-targeted organisms. For this reason, compositions including *Bacillus thuringiensis* strains or their insecticidal proteins can be used as environmentally acceptable insecticides to control agricultural insect pests or insect vectors for a variety of human or animal diseases.

15 Crystal (Cry) proteins (delta-endotoxins) from *Bacillus thuringiensis* have potent insecticidal activity against predominantly Lepidopteran, Dipteran, and Coleopteran larvae. These proteins also have shown activity against Hymenoptera, Homoptera, Phthiraptera, Mallophaga, and Acari pest orders, as well as other invertebrate orders such as Nemathelminthes, Platyhelminthes, and Sarcomastigophora (Feitelson (1993) The 20 Bacillus Thuringiensis family tree. *In Advanced Engineered Pesticides*. Marcel Dekker, Inc., New York, N.Y.) These proteins were originally classified as CryI to CryV based

primarily on their insecticidal activity. The major classes were Lepidoptera-specific (I), Lepidoptera- and Diptera-specific (II), Coleoptera-specific (III), Diptera-specific (IV), and nematode-specific (V) and (VI). The proteins were further classified into subfamilies; more highly related proteins within each family were assigned divisional letters such as Cry1A, Cry1B, Cry1C, etc. Even more closely related proteins within each division were given names such as Cry1C1, Cry1C2, etc.

5 A new nomenclature was recently described for the Cry genes based upon amino acid sequence homology rather than insect target specificity (Crickmore *et al.* (1998) *Microbiol. Mol. Biol. Rev.* 62:807-813). In the new classification, each toxin is assigned
10 a unique name incorporating a primary rank (an Arabic number), a secondary rank (an uppercase letter), a tertiary rank (a lowercase letter), and a quaternary rank (another Arabic number). In the new classification, Roman numerals have been exchanged for Arabic numerals in the primary rank. Proteins with less than 45% sequence identity have different primary ranks, and the criteria for secondary and tertiary ranks are 78% and
15 95%, respectively.

The crystal protein does not exhibit insecticidal activity until it has been ingested and solubilized in the insect midgut. The ingested protoxin is hydrolyzed by proteases in the insect digestive tract to an active toxic molecule. (Höfte and Whiteley (1989) *Microbiol. Rev.* 53:242-255). This toxin binds to apical brush border receptors in the
20 midgut of the target larvae and inserts into the apical membrane creating ion channels or pores, resulting in larval death.

Delta-endotoxins generally have five conserved sequence domains, and three conserved structural domains (see, for example, de Maagd *et al.* (2001) *Trends Genetics* 17:193-199). The first conserved structural domain consists of seven alpha helices and is
25 involved in membrane insertion and pore formation. Domain II consists of three beta-sheets arranged in a Greek key configuration, and domain III consists of two antiparallel beta-sheets in 'jelly-roll' formation (de Maagd *et al.* (2001) *supra*). Domains II and III are involved in receptor recognition and binding, and are therefore considered determinants of toxin specificity.

30 Because of the devastation that insects can confer, there is a continual need to discover new forms of *Bacillus thuringiensis* delta-endotoxins.

SUMMARY OF INVENTION

Compositions and methods for conferring pesticide resistance to bacteria, plants, plant cells, tissues, and seeds are provided. Compositions include isolated nucleic acid molecules encoding sequences for delta-endotoxin and delta-endotoxin-associated polypeptides, vectors comprising those nucleic acid molecules, and host cells comprising the vectors. Compositions also include isolated or recombinant polypeptide sequences of the endotoxin, compositions comprising these polypeptides, and antibodies to those polypeptides. The nucleotide sequences can be used in DNA constructs or expression cassettes for transformation and expression in organisms, including microorganisms and plants. The nucleotide or amino acid sequences may be synthetic sequences that have been designed for optimum expression in an organism, including, but not limited to, a microorganism or a plant. Compositions also comprise transformed bacteria, plants, plant cells, tissues, and seeds.

In particular, the present invention provides for isolated nucleic acid molecules comprising a nucleotide sequence encoding an amino acid sequence shown in SEQ ID NO:3, 5, or 7, or a nucleotide sequence set forth in SEQ ID NO:1, 2, 4, or 6, as well as variants and fragments thereof. Nucleotide sequences that are complementary to a nucleotide sequence of the invention, or that hybridize to a sequence of the invention, are also encompassed.

Methods are provided for producing the polypeptides of the invention, and for using those polypeptides for controlling or killing a lepidopteran or coleopteran pest.

The compositions and methods of the invention are useful for the production of organisms with pesticide resistance, specifically bacteria and plants. These organisms and compositions derived from them are desirable for agricultural purposes. The compositions of the invention are also useful for generating altered or improved delta-endotoxin or delta-endotoxin-associated proteins that have pesticidal activity, or for detecting the presence of delta-endotoxin or delta-endotoxin-associated proteins or nucleic acids in products or organisms.

DESCRIPTION OF FIGURES

Figures 1A, B, and C show an alignment of AXMI-008 (SEQ ID NO:3) with cry1Aa (SEQ ID NO:8), cry1Ac (SEQ ID NO:9), cry1Ia (SEQ ID NO:10), cry2Aa (SEQ ID NO:11), cry3Aa1 (SEQ ID NO:12), cry3Bb (SEQ ID NO:13), cry4Aa (SEQ ID NO:14),
5 cry4Ba (SEQ ID NO:15), cry6Aa (SEQ ID NO:16), cry7Aa (SEQ ID NO:17), cry8Aa (SEQ ID NO:18), cry10Aa (SEQ ID NO:19), cry16Aa (SEQ ID NO:20), cry19Ba (SEQ ID NO:21), cry24Aa (SEQ ID NO:22), cry25Aa (SEQ ID NO:23), cry39Aa1 (SEQ ID NO:24), and cry40Aa1 (SEQ ID NO:25). Toxins having C-terminal non-toxic domains were artificially truncated as shown. The alignment shows the most highly conserved
10 amino acid residues highlighted in black, and highly conserved amino acid residues highlighted in gray. Conserved group 1 is found from about amino acid residue 185 to about 206 of SEQ ID NO:3. Conserved group 2 is found from about amino acid residue 276 to about 318 of SEQ ID NO:3. Conserved group 3 is found from about amino acid residue 497 to about 547 of SEQ ID NO:3. Conserved group 4 is found from about
15 amino acid residue 576 to about 586 of SEQ ID NO:3. Conserved group 5 is found from about amino acid residue 657 to about 667 of SEQ ID NO:3

Figures 2A and B show an alignment of AXMI-008orf2 (SEQ ID NO:7) with cry19Aa-orf2 (SEQ ID NO:26), crybun2-orf2 (SEQ ID NO:27), crybun3-orf2 (SEQ ID NO:28),
20 cry4Aa (SEQ ID NO:14), and cry4Ba (SEQ ID NO:15). The alignment shows the most highly conserved amino acid residues highlighted in black, and highly conserved amino acid residues highlighted in gray.

DETAILED DESCRIPTION

25 The present invention is drawn to compositions and methods for regulating pest resistance in organisms, particularly plants or plant cells. The methods involve transforming organisms with a nucleotide sequence encoding a delta-endotoxin or delta-endotoxin-associated protein of the invention. In particular, the nucleotide sequences of the invention are useful for preparing plants and microorganisms that possess pesticidal activity. Thus, transformed bacteria, plants, plant cells, plant tissues and seeds are provided. Compositions are delta-endotoxin or delta-endotoxin-associated nucleic acids
30

and proteins of *Bacillus thuringiensis*. The sequences find use in the construction of expression vectors for subsequent transformation into organisms of interest, as probes for the isolation of other delta-endotoxin or delta-endotoxin-associated genes, and for the generation of altered pesticidal proteins by methods known in the art, such as domain swapping or DNA shuffling. The proteins find use in controlling or killing lepidopteran or coleopteran pest populations and for producing compositions with pesticidal activity.

Definitions

By "delta-endotoxin" is intended a toxin from *Bacillus thuringiensis* that has toxic activity against one or more pests, including, but not limited to, members of the Lepidoptera, Diptera, and Coleoptera orders. In some cases, delta-endotoxin proteins have been isolated from other organisms, including *Clostridium bifermentans* and *Paenibacillus popilliae*. Delta-endotoxin proteins include amino acid sequences deduced from the full-length nucleotide sequences disclosed herein, and amino acid sequences that are shorter than the full-length sequences, either due to the use of an alternate downstream start site, or due to processing that produces a shorter protein having pesticidal activity. Processing may occur in the organism the protein is expressed in, or in the pest after ingestion of the protein. Delta-endotoxins include proteins identified as cry1 through cry43, cyt1 and cyt2, and Cyt-like toxin. There are currently over 250 known species of delta-endotoxins with a wide range of specificities and toxicities. For an expansive list see Crickmore *et al.* (1998), *Microbiol. Mol. Biol. Rev.* 62:807-813, and for regular updates see Crickmore *et al.* (2003) "Bacillus thuringiensis toxin nomenclature," at www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.

Bacterial genes, such as the AXMI-008 gene of this invention, quite often possess multiple methionine initiation codons in proximity to the start of the open reading frame. Often, translation initiation at one or more of these start codons will lead to generation of a functional protein. These start codons can include ATG codons. However, bacteria such as *Bacillus sp.* also recognize the codon GTG as a start codon, and proteins that initiate translation at GTG codons contain a methionine at the first amino acid. Furthermore, it is not often determined *a priori* which of these codons are used naturally in the bacterium. Thus, it is understood that use of one of the alternate methionine codons may also lead to

generation of delta-endotoxin proteins that encode pesticidal activity. For example, an alternate start site for a delta-endotoxin protein of the invention may be at nucleotide 177 of SEQ ID NO:1. Translation from this alternate start site results in the amino acid sequence found in SEQ ID NO:5. These delta-endotoxin proteins are encompassed in the 5 present invention and may be used in the methods of the present invention.

In addition, there may be one or more additional open reading frames in the disclosed nucleotide sequences that encode one or more delta-endotoxin-associated proteins. By “delta-endotoxin-associated protein” is intended a protein encoded by a nucleotide sequence disclosed herein using an alternate open reading frame than that used 10 by the delta-endotoxins of the present invention. Proteins such as these are known in the art as helper proteins, stabilizing sequences, or delta-endotoxin-associated proteins. These delta-endotoxin-associated proteins may have pesticidal activity, or may be 15 important in facilitating expression of delta-endotoxin proteins. Methods are known in the art for measuring pesticidal activity and for determining the effects of delta-endotoxin-associated proteins on delta-endotoxin protein expression and crystal formation (see, for example, Park *et al.* (1999) *FEMS Microbiol. Lett.* 181:319-327; Ge *et al.* (1998) *FEMS Microbiol. Lett.* 165:35-41; Rosso and Delecluse (1997) *Appl. Environ. Microbiol.* 63:4449-4455). These delta-endotoxin-associated proteins are encompassed by the present invention, and may be used in the methods disclosed herein, either alone or 20 in combination with known delta-endotoxin proteins. In one embodiment, the delta-endotoxin-associated protein has the amino acid sequence found in SEQ ID NO:7 and is encoded by the nucleotide sequence of SEQ ID NO:6.

By “plant cell” is intended all known forms of plant, including undifferentiated tissue (e.g. callus), suspension culture cells, protoplasts, leaf cells, root cells, phloem ~ 25 cells, plant seeds, pollen, propagules, embryos and the like. By “plant expression cassette” is intended a DNA construct that is capable of resulting in the expression of a protein from an open reading frame in a plant cell. Typically these contain a promoter and a coding sequence. Often, such constructs will also contain a 3' untranslated region. Such constructs may contain a ‘signal sequence’ or ‘leader sequence’ to facilitate co- 30 translational or post-translational transport of the peptide to certain intracellular structures such as the chloroplast (or other plastid), endoplasmic reticulum, or Golgi apparatus.

By “signal sequence” is intended a sequence that is known or suspected to result in cotranslational or post-translational peptide transport across the cell membrane. In eukaryotes, this typically involves secretion into the Golgi apparatus, with some resulting glycosylation. By “leader sequence” is intended any sequence that when translated, 5 results in an amino acid sequence sufficient to trigger co-translational transport of the peptide chain to a sub-cellular organelle. Thus, this includes leader sequences targeting transport and/or glycosylation by passage into the endoplasmic reticulum, passage to vacuoles, plastids including chloroplasts, mitochondria, and the like.

By “plant transformation vector” is intended a DNA molecule that is necessary 10 for efficient transformation of a plant cell. Such a molecule may consist of one or more plant expression cassettes, and may be organized into more than one ‘vector’ DNA molecule. For example, binary vectors are plant transformation vectors that utilize two non-contiguous DNA vectors to encode all requisite cis- and trans-acting functions for transformation of plant cells (Hellens and Mullineaux (2000) *Trends in Plant Science* 15 5:446-451). “Vector” refers to a nucleic acid construct designed for transfer between different host cells. “Expression vector” refers to a vector that has ability to incorporate, integrate and express heterologous DNA sequences or fragments in a foreign cell.

“Transgenic plants” or “transformed plants” or “stably transformed plants or cells 20 or tissues” refers to plants that have incorporated or integrated exogenous nucleic acid sequences or DNA fragments into the plant cell. These nucleic acid sequences include those that are exogenous, or not present in the untransformed plant cell, as well as those that may be endogenous, or present in the untransformed plant cell. “Heterologous” generally refers to the nucleic acid sequences that are not endogenous to the cell or part 25 of the native genome in which they are present, and have been added to the cell by infection, transfection, microinjection, electroporation, microprojection, or the like.

“Promoter” refers to a nucleic acid sequence that functions to direct transcription of a downstream coding sequence. The promoter together with other transcriptional and translational regulatory nucleic acid sequences (also termed “control sequences”) are necessary for the expression of a DNA sequence of interest.

30 Provided herein are novel isolated nucleotide sequences that confer pesticidal activity. Also provided are the amino acid sequences for the delta-endotoxin and delta-

endotoxin-associated proteins. The protein resulting from translation of this gene allows cells to control or kill pests that ingest it.

An "isolated" or "purified" nucleic acid molecule or protein, or biologically active portion thereof, is substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Preferably, an "isolated" nucleic acid is free of sequences (preferably protein encoding sequences) that naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For purposes of the invention,

10 "isolated" when used to refer to nucleic acid molecules excludes isolated chromosomes.

For example, in various embodiments, the isolated delta-endotoxin or delta-endotoxin-associated-encoding nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequence that naturally flanks the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. A delta-endotoxin or delta-endotoxin-associated protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, or 5% (by dry weight) of non-delta-endotoxin or non-delta-endotoxin-associated protein (also referred to herein as a "contaminating protein"). Various aspects of the invention are described in further detail in the following subsections.

20

Isolated Nucleic Acid Molecules, and Variants and Fragments Thereof

One aspect of the invention pertains to isolated nucleic acid molecules comprising nucleotide sequences encoding delta-endotoxin or delta-endotoxin-associated proteins and polypeptides or biologically active portions thereof, as well as nucleic acid molecules sufficient for use as hybridization probes to identify delta-endotoxin or delta-endotoxin-associated-encoding nucleic acids. As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

Nucleotide sequences encoding the proteins of the present invention include the sequences set forth in SEQ ID NOS:1, 2, 4, and 6, and complements thereof. By "complement" is intended a nucleotide sequence that is sufficiently complementary to a given nucleotide sequence such that it can hybridize to the given nucleotide sequence to thereby form a stable duplex. The corresponding amino acid sequences for the delta-endotoxin or delta-endotoxin-associated proteins encoded by these nucleotide sequences are set forth in SEQ ID NOS:3, 5, and 7.

Nucleic acid molecules that are fragments of these delta-endotoxin or delta-endotoxin-associated protein-encoding nucleotide sequences are also encompassed by the present invention. By "fragment" is intended a portion of the nucleotide sequence encoding a delta-endotoxin protein or delta-endotoxin-associated protein. A fragment of a nucleotide sequence may encode a biologically active portion of a delta-endotoxin or delta-endotoxin-associated protein, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. Nucleic acid molecules that are fragments of a delta-endotoxin or a delta-endotoxin-associated nucleotide sequence comprise at least about 15, 20, 50, 75, 100, 200, 300, 350, 400, 450, 500, 550, 600, 650, 700, 750, 800, 850, 900, 950, 1000, 1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900, 2000, 2100, 2200, 2300, 2400, 2500, 3000, 3500, 4000, 4500, 5000, 5500 nucleotides, or up to the number of nucleotides present in a full-length delta-endotoxin or delta-endotoxin-associated protein-encoding nucleotide sequence disclosed herein (for example, 5980 nucleotides for SEQ ID NO:1, 2082 for SEQ ID NO:2, 2073 for SEQ ID NO:4, or 1686 for SEQ ID NO:6), depending upon the intended use.

Fragments of the nucleotide sequences of the present invention will encode protein fragments that retain the biological activity of the delta-endotoxin or delta-endotoxin-associated protein and, hence, retain pesticidal activity or delta-endotoxin-associated protein activity, respectively. By "delta-endotoxin activity" is intended pesticidal activity. By "delta-endotoxin-associated protein activity" is intended that the protein have pesticidal activity, or that the protein improves expression of a delta-endotoxin protein. This improvement in protein expression can happen by any mechanism. By "retains activity" is intended that the fragment will have at least about 30%, preferably at least about 50%, more preferably at least about 70%, even more

preferably at least about 80% of the activity of the delta-endotoxin or delta-endotoxin-associated protein. Methods are known in the art for determining the effects of delta-endotoxin-associated proteins on delta-endotoxin protein expression and crystal formation (see, for example, Park *et al.* (1999) *FEMS Microbiol. Lett.* 181:319-327; Ge *et al.* (1998) *FEMS Microbiol. Lett.* 165:35-41; Rosso and Delecluse (1997) *Appl. Environ. Microbiol.* 63:4449-4455). Methods for measuring pesticidal activity are well known in the art. See, for example, Czapla and Lang (1990) *J. Econ. Entomol.* 83(6): 2480-2485; Andrews *et al.* (1988) *Biochem. J.* 252:199-206; Marrone *et al.* (1985) *J. of Economic Entomology* 78:290-293; and U.S. Patent No. 5,743,477, all of which are herein 10 incorporated by reference in their entirety.

A fragment of a delta-endotoxin or delta-endotoxin-associated protein-encoding nucleotide sequence that encodes a biologically active portion of a protein of the invention will encode at least about 15, 25, 30, 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, 400, 450, 500, 550, 600, or 650 contiguous amino acids, or up to the total number of 15 amino acids present in a full-length delta-endotoxin or delta-endotoxin-associated protein of the invention (for example, 693 amino acids for SEQ ID NO:3, 690 amino acids for SEQ ID NO:5, or 561 amino acids for SEQ ID NO:7).

Preferred delta-endotoxin or delta-endotoxin-associated proteins of the present invention are encoded by a nucleotide sequences sufficiently identical to the nucleotide sequences of SEQ ID NO:1, 2, 4, or 6. By "sufficiently identical" is intended an amino acid or nucleotide sequence that has at least about 60% or 65% sequence identity, preferably about 70% or 75% sequence identity, more preferably about 80% or 85% sequence identity, most preferably about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity compared to a reference sequence using one of the 20 alignment programs described herein using standard parameters. One of skill in the art will recognize that these values can be appropriately adjusted to determine corresponding 25 identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning, and the like.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes. The percent identity 30 between the two sequences is a function of the number of identical positions shared by

the sequences (i.e., percent identity = number of identical positions/total number of positions (e.g., overlapping positions) x 100). In one embodiment, the two sequences are the same length. The percent identity between two sequences can be determined using techniques similar to those described below, with or without allowing gaps. In 5 calculating percent identity, typically exact matches are counted.

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A nonlimiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264, modified as in Karlin and Altschul 10 (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the BLASTN and BLASTX programs of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403. BLAST nucleotide searches can be performed with the BLASTN program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to delta-endotoxin or delta-endotoxin-associated nucleic acid molecules of the invention. BLAST protein searches 15 can be performed with the BLASTX program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to delta-endotoxin or delta-endotoxin-associated protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-Blast can be used to perform an iterated search 20 that detects distant relationships between molecules. See, Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., BLASTX and BLASTN) can be used. See, www.ncbi.nlm.nih.gov. Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the ClustalW algorithm (Higgins *et al.* (1994) 25 *Nucleic Acids Res.* 22:4673-4680). ClustalW compares sequences and aligns the entirety of the amino acid or DNA sequence, and thus can provide data about the sequence conservation of the entire amino acid sequence. The ClustalW algorithm is used in several commercially available DNA/amino acid analysis software packages, such as the ALIGNX module of the vector NTi Program Suite (Informax, Inc). After alignment of 30 amino acid sequences with ClustalW, the percent amino acid identity can be assessed. A non-limiting example of a software program useful for analysis of ClustalW alignments is

GeneDoc™. Genedoc™ (Karl Nicholas) allows assessment of amino acid (or DNA) similarity and identity between multiple proteins. Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17. Such an algorithm is incorporated into the 5 ALIGN program (version 2.0), which is part of the GCG sequence alignment software package (available from Accelrys, Inc., 9865 Scranton Rd., San Diego, California, USA). When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

The invention also encompasses variant nucleic acid molecules. "Variants" of the 10 delta-endotoxin or delta-endotoxin-associated protein-encoding nucleotide sequences include those sequences that encode the delta-endotoxin or delta-endotoxin-associated proteins disclosed herein but that differ conservatively because of the degeneracy of the genetic code as well as those that are sufficiently identical as discussed above. Naturally occurring allelic variants can be identified with the use of well-known molecular biology 15 techniques, such as polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant nucleotide sequences also include synthetically derived nucleotide sequences that have been generated, for example, by using site-directed mutagenesis but which still encode the delta-endotoxin or delta-endotoxin-associated proteins disclosed in the present invention as discussed below. Variant proteins 20 encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein, that is, retaining pesticidal activity. By "retains activity" is intended that the variant will have at least about 30%, preferably at least about 50%, more preferably about at least 70%, even more preferably at least about 80% of the activity of the native protein. Methods for measuring pesticidal 25 activity are well known in the art. See, for example, Czapla and Lang (1990) *J. Econ. Entomol.* 83(6): 2480-2485; Andrews *et al.* (1988) *Biochem. J.* 252:199-206; Marrone *et al.* (1985) *J. of Economic Entomology* 78:290-293; and U.S. Patent No. 5,743,477, all of which are herein incorporated by reference in their entirety.

The invention also encompasses variant nucleic acid molecules. "Variants" of the 30 delta-endotoxin or delta-endotoxin-associated-encoding nucleotide sequences include those sequences that encode the delta-endotoxin or delta-endotoxin-associated proteins

disclosed herein but that differ conservatively because of the degeneracy of the genetic code as well as those that are sufficiently identical as discussed above. Naturally occurring allelic variants can be identified with the use of well-known molecular biology techniques, such as polymerase chain reaction (PCR) and hybridization techniques as 5 outlined below. Variant nucleotide sequences also include synthetically derived nucleotide sequences that have been generated, for example, by using site-directed mutagenesis but which still encode the delta-endotoxin or delta-endotoxin-associated proteins disclosed in the present invention as discussed below.

The skilled artisan will further appreciate that changes can be introduced by 10 mutation into the nucleotide sequences of the invention thereby leading to changes in the amino acid sequence of the encoded delta-endotoxin or delta-endotoxin-associated proteins, without altering the biological activity of the proteins. Thus, variant isolated nucleic acid molecules can be created by introducing one or more nucleotide substitutions, additions, or deletions into the corresponding nucleotide sequence disclosed 15 herein, such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations can be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Such variant nucleotide sequences are also encompassed by the present invention.

For example, preferably, conservative amino acid substitutions may be made at 20 one or more predicted, preferably nonessential amino acid residues. A "nonessential" amino acid residue is a residue that can be altered from the wild-type sequence of a delta-endotoxin or delta-endotoxin-associated protein without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. A "conservative amino acid substitution" is one in which the amino acid residue is replaced 25 with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, 30 valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-

branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

There are generally five highly conserved regions among the delta-endotoxin proteins, concentrated largely in the center of the domain or at the junction between 5 domains (Rajamohan *et al.* (1998) *Prog. Nucleic Acid Res. Mol. Biol.* 60:1 –23). The blocks of conserved amino acids for various delta-endotoxins as well as consensus sequences may be found in Schnepf *et al.* (1998) *Microbio. Mol. Biol. Rev.* 62:775-806 and Lereclus *et al.* (1989) Role, Structure, and Molecular Organization of the Genes Coding for the Parasporal d-endotoxins of *Bacillus thuringiensis*. In Regulation of 10 Prokaryotic Development. Issar Smit, Slepecky, R.A., Setlow, P. American Society for Microbiology, Washington, D.C. 20006. It has been proposed that delta-endotoxins having these conserved regions may share a similar structure, consisting of three domains (Li *et al.* (1991) *Nature* 353: 815-821). Domain I has the highest similarity between delta-endotoxins (Bravo (1997) *J. Bacteriol.* 179:2793-2801).

15 Amino acid substitutions may be made in nonconserved regions that retain function. In general, such substitutions would not be made for conserved amino acid residues, or for amino acid residues residing within a conserved motif, where such residues are essential for protein activity. Examples of residues that are conserved and that may be essential for protein activity include, for example, residues that are identical 20 between all proteins contained in the alignment of Figures 1A, B, and C or 2A and B. Examples of residues that are conserved but that may allow conservative amino acid substitutions and still retain activity include, for example, residues that have only 25 conservative substitutions between all proteins contained in the alignment of Figures 1A, B, and C or 2A and B. However, one of skill in the art would understand that functional variants may have minor conserved or nonconserved alterations in the conserved residues.

Alternatively, variant nucleotide sequences can be made by introducing mutations 30 randomly along all or part of the coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for ability to confer delta-endotoxin or delta-endotoxin-associated activity to identify mutants that retain activity. Following

mutagenesis, the encoded protein can be expressed recombinantly, and the activity of the protein can be determined using standard assay techniques.

Using methods such as PCR, hybridization, and the like corresponding delta-endotoxin or delta-endotoxin-associated sequences can be identified, such sequences 5 having substantial identity to the sequences of the invention. *See*, for example, Sambrook J., and Russell, D.W. (2001) *Molecular Cloning: A Laboratory Manual*. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) and Innis, *et al.* (1990) *PCR Protocols: A Guide to Methods and Applications* (Academic Press, NY).

In a hybridization method, all or part of the delta-endotoxin or delta-endotoxin-associated nucleotide sequence can be used to screen cDNA or genomic libraries. 10 Methods for construction of such cDNA and genomic libraries are generally known in the art and are disclosed in Sambrook and Russell, 2001. The so-called hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may be labeled with a detectable group such as ³²P, or any other 15 detectable marker, such as other radioisotopes, a fluorescent compound, an enzyme, or an enzyme co-factor. Probes for hybridization can be made by labeling synthetic oligonucleotides based on the known delta-endotoxin or delta-endotoxin-associated-encoding nucleotide sequence disclosed herein. Degenerate primers designed on the basis of conserved nucleotides or amino acid residues in the nucleotide sequence or 20 encoded amino acid sequence can additionally be used. The probe typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, preferably about 25, more preferably at least about 50, 75, 100, 125, 150, 175, 200, 250, 300, 350, or 400 consecutive nucleotides of delta-endotoxin or delta-endotoxin-associated-encoding nucleotide sequence of the invention or a fragment or variant 25 thereof. Preparation of probes for hybridization is generally known in the art and is disclosed in Sambrook and Russell, 2001, herein incorporated by reference.

In hybridization techniques, all or part of a known nucleotide sequence is used as a probe that selectively hybridizes to other corresponding nucleotide sequences present in a population of cloned genomic DNA fragments or cDNA fragments (i.e., genomic or 30 cDNA libraries) from a chosen organism. The hybridization probes may be genomic DNA fragments, cDNA fragments, RNA fragments, or other oligonucleotides, and may

be labeled with a detectable group such as ^{32}P , or any other detectable marker. Thus, for example, probes for hybridization can be made by labeling synthetic oligonucleotides based on the delta-endotoxin or delta-endotoxin-associated sequence of the invention. Methods for preparation of probes for hybridization and for construction of cDNA and 5 genomic libraries are generally known in the art and are disclosed in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

For example, the entire delta-endotoxin or delta-endotoxin-associated sequence disclosed herein, or one or more portions thereof, may be used as a probe capable of 10 specifically hybridizing to corresponding delta-endotoxin or delta-endotoxin-associated-like sequences and messenger RNAs. To achieve specific hybridization under a variety of conditions, such probes include sequences that are unique and are preferably at least about 10 nucleotides in length, and most preferably at least about 20 nucleotides in length. Such probes may be used to amplify corresponding delta-endotoxin or delta- 15 endotoxin-associated sequences from a chosen organism by PCR. This technique may be used to isolate additional coding sequences from a desired organism or as a diagnostic assay to determine the presence of coding sequences in an organism. Hybridization techniques include hybridization screening of plated DNA libraries (either plaques or colonies; see, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory 20 Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

Hybridization of such sequences may be carried out under stringent conditions. By "stringent conditions" or "stringent hybridization conditions" is intended conditions under which a probe will hybridize to its target sequence to a detectably greater degree than to other sequences (e.g., at least 2-fold over background). Stringent conditions are 25 sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences that are 100% complementary to the probe can be identified (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is 30 less than about 1000 nucleotides in length, preferably less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g., 10 to 50 nucleotides) and at least about 60°C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37°C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55°C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1.0 M NaCl, 1% SDS at 37°C, and a wash in 0.5X to 1X SSC at 55 to 60°C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 0.1X SSC at 60 to 65°C. Optionally, wash buffers may comprise about 0.1% to about 1% SDS. Duration of hybridization is generally less than about 24 hours, usually about 4 to about 12 hours.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284: $T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{form}) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq 90\%$ identity are sought, the T_m can be decreased 10°C. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4°C lower than the thermal melting point (T_m); moderately stringent conditions can

utilize a hybridization and/or wash at 6, 7, 8, 9, or 10°C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20°C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will

5 understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45°C (aqueous solution) or 32°C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in*

10 *Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 (Elsevier, New York); and Ausubel *et al.*, eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, New York).

15

Isolated Proteins and Variants and Fragments Thereof

Delta-endotoxin and delta-endotoxin-associated proteins are also encompassed within the present invention. By "delta-endotoxin protein" is intended a protein having the amino acid sequence set forth in SEQ ID NO:3 or 5. By "delta-endotoxin-associated protein" is intended a protein having the amino acid sequence set forth in SEQ ID NO:7. Fragments, biologically active portions, and variants thereof are also provided, and may be used to practice the methods of the present invention.

20

"Fragments" or "biologically active portions" include polypeptide fragments comprising a portion of an amino acid sequence encoding a delta-endotoxin or delta-endotoxin-associated protein as set forth in SEQ ID NO:3, 5, or 7, and that retain delta-endotoxin activity or delta-endotoxin-associated activity. A biologically active portion of a delta-endotoxin or delta-endotoxin-associated protein can be a polypeptide that is, for example, 10, 25, 50, 100 or more amino acids in length. Such biologically active portions can be prepared by recombinant techniques and evaluated for delta-endotoxin or delta-endotoxin-associated activity. Methods for measuring pesticidal activity are well known in the art. See, for example, Czapla and Lang (1990) *J. Econ. Entomol.* 83(6):

25

2480-2485; Andrews *et al.* (1988) *Biochem. J.* 252:199-206; Marrone *et al.* (1985) *J. of Economic Entomology* 78:290-293; and U.S. Patent No. 5,743,477, all of which are herein incorporated by reference in their entirety. As used here, a fragment comprises at least 8 contiguous amino acids SEQ ID NO:3, 5, or 7. The invention encompasses other 5 fragments, however, such as any fragment in the protein greater than about 10, 20, 30, 50, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, and 650 amino acids.

By "variants" is intended proteins or polypeptides having an amino acid sequence that is at least about 60%, 65%, preferably about 70%, 75%, more preferably about 80%, 85%, most preferably about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% 10 identical to the amino acid sequence of SEQ ID NO:3, 5, or 7. Variants also include polypeptides encoded by a nucleic acid molecule that hybridizes to the nucleic acid molecule of SEQ ID NO:1, 2, 4, or 6, or a complement thereof, under stringent conditions. Such variants generally retain delta-endotoxin or delta-endotoxin-associated activity. Variants include polypeptides that differ in amino acid sequence due to 15 mutagenesis. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein, that is, retaining pesticidal activity. Methods for measuring pesticidal activity are well known in the art. See, for example, Czapla and Lang (1990) *J. Econ. Entomol.* 83(6): 2480-2485; Andrews *et al.* (1988) *Biochem. J.* 252:199-206; Marrone *et al.* (1985) *J. of Economic Entomology* 78:290-293; and U.S. Patent No. 5,743,477, all of which are 20 herein incorporated by reference in their entirety.

Altered or Improved Variants

It is recognized that DNA sequences of a delta-endotoxin or delta-endotoxin-associated protein may be altered by various methods, and that these alterations may 25 result in DNA sequences encoding proteins with amino acid sequences different than that encoded by the delta-endotoxin or delta-endotoxin-associated protein of the present invention. This protein may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions. Methods for such manipulations are generally known in the art. For example, amino acid sequence variants of the delta-endotoxin or delta-endotoxin-associated protein can be prepared by mutations in the 30

DNA. This may also be accomplished by one of several forms of mutagenesis and/or in directed evolution. In some aspects, the changes encoded in the amino acid sequence will not substantially affect the function of the protein. Such variants will possess the desired pesticidal activity. However, it is understood that the ability of a delta-endotoxin or delta-
5 endotoxin-associated protein to confer pesticidal activity may be improved by the use of such techniques upon the compositions of this invention. For example, one may express the delta-endotoxin or delta-endotoxin-associated protein in host cells that exhibit high rates of base misincorporation during DNA replication, such as XL-1 Red (Stratagene). After propagation in such strains, one can isolate the delta-endotoxin or delta-endotoxin-
10 associated DNA (for example by preparing plasmid DNA, or by amplifying by PCR and cloning the resulting PCR fragment into a vector), culture the delta-endotoxin or delta-endotoxin-associated mutations in a non-mutagenic strain, and identify mutated delta-endotoxin or delta-endotoxin-associated genes with pesticidal activity, for example by performing an assay to test for pesticidal activity. Generally, the protein is mixed and
15 used in feeding assays. See, for example Marrone *et al.* (1985) *J. of Economic Entomology* 78:290-293. Such assays can include contacting plants with one or more pests and determining the plant's ability to survive and/or cause the death of the pests. Examples of mutations that result in increased toxicity are found in Schnepf *et al.* (1998) *Microbiol. Mol. Biol. Rev.* 62:775-806.

20 Alternatively, alterations may be made to the protein sequence of many proteins at the amino or carboxy terminus without substantially affecting activity. This can include insertions, deletions, or alterations introduced by modern molecular methods, such as PCR, including PCR amplifications that alter or extend the protein coding sequence by virtue of inclusion of amino acid encoding sequences in the oligonucleotides
25 utilized in the PCR amplification. Alternatively, the protein sequences added can include entire protein-coding sequences, such as those used commonly in the art to generate protein fusions. Such fusion proteins are often used to (1) increase expression of a protein of interest (2) introduce a binding domain, enzymatic activity, or epitope to facilitate either protein purification, protein detection, or other experimental uses known in the art
30 (3) target secretion or translation of a protein to a subcellular organelle, such as the

periplasmic space of Gram-negative bacteria, or the endoplasmic reticulum of eukaryotic cells, the latter of which often results in glycosylation of the protein.

Variant nucleotide and amino acid sequences of the present invention also encompass sequences derived from mutagenic and recombinogenic procedures such as 5 DNA shuffling. With such a procedure, one or more different delta-endotoxin or delta-endotoxin-associated protein coding regions can be used to create a new delta-endotoxin or delta-endotoxin-associated protein possessing the desired properties. In this manner, libraries of recombinant polynucleotides are generated from a population of related sequence polynucleotides comprising sequence regions that have substantial sequence 10 identity and can be homologously recombined *in vitro* or *in vivo*. For example, using this approach, sequence motifs encoding a domain of interest may be shuffled between the delta-endotoxin or delta-endotoxin-associated gene of the invention and other known delta-endotoxin or delta-endotoxin-associated genes to obtain a new gene coding for a protein with an improved property of interest, such as an increased insecticidal activity.

15 Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751; Stemmer (1994) *Nature* 370:389-391; Crameri *et al.* (1997) *Nature Biotech.* 15:436-438; Moore *et al.* (1997) *J. Mol. Biol.* 272:336-347; Zhang *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:4504-4509; Crameri *et al.* (1998) *Nature* 391:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

20 Domain swapping or shuffling is another mechanism for generating altered delta-endotoxin or delta-endotoxin-associated proteins. Domains II and III may be swapped between delta-endotoxin proteins, resulting in hybrid or chimeric toxins with improved pesticidal activity or target spectrum. Methods for generating recombinant proteins and testing them for pesticidal activity are well known in the art (see, for example, Naimov *et al.* (2001) *Appl. Environ. Microbiol.* 67:5328-5330; de Maagd *et al.* (1996) *Appl. Environ. Microbiol.* 62:1537-1543; Ge *et al.* (1991) *J. Biol. Chem.* 266:17954-17958; Schnepf *et al.* (1990) *J. Biol. Chem.* 265:20923-20930; Rang *et al.* 91999) *Appl. Environ. Microbiol.* 65:2918-2925).

Plant Transformation

Transformation of plant cells can be accomplished by one of several techniques known in the art. First, one engineers the delta-endotoxin or delta-endotoxin-associated gene in a way that allows its expression in plant cells. Typically a construct that expresses 5 such a protein would contain a promoter to drive transcription of the gene, as well as a 3' untranslated region to allow transcription termination and polyadenylation. The organization of such constructs is well known in the art. In some instances, it may be useful to engineer the gene such that the resulting peptide is secreted, or otherwise targeted within the plant cell. For example, the gene can be engineered to contain a signal 10 peptide to facilitate transfer of the peptide to the endoplasmic reticulum. It may also be preferable to engineer the plant expression cassette to contain an intron, such that mRNA processing of the intron is required for expression.

Typically this 'plant expression cassette' will be inserted into a 'plant transformation vector'. This plant transformation vector may be comprised of one or 15 more DNA vectors needed for achieving plant transformation. For example, it is a common practice in the art to utilize plant transformation vectors that are comprised of more than one contiguous DNA segment. These vectors are often referred to in the art as 'binary vectors'. Binary vectors as well as vectors with helper plasmids are most often used for *Agrobacterium*-mediated transformation, where the size and complexity of DNA 20 segments needed to achieve efficient transformation is quite large, and it is advantageous to separate functions onto separate DNA molecules. Binary vectors typically contain a plasmid vector that contains the cis-acting sequences required for T-DNA transfer (such as left border and right border), a selectable marker that is engineered to be capable of expression in a plant cell, and a 'gene of interest' (a gene engineered to be capable of expression in a plant cell for which generation of transgenic plants is desired). Also 25 present on this plasmid vector are sequences required for bacterial replication. The cis-acting sequences are arranged in a fashion to allow efficient transfer into plant cells and expression therein. For example, the selectable marker gene and the gene of interest are located between the left and right borders. Often a second plasmid vector contains the trans-acting factors that mediate T-DNA transfer from *Agrobacterium* to plant cells. This 30 plasmid often contains the virulence functions (Vir genes) that allow infection of plant

cells by *Agrobacterium*, and transfer of DNA by cleavage at border sequences and virus-mediated DNA transfer, as is understood in the art (Hellens and Mullineaux (2000) *Trends in Plant Science*, 5:446-451). Several types of *Agrobacterium* strains (e.g. LBA4404, GV3101, EHA101, EHA105, etc.) can be used for plant transformation. The 5 second plasmid vector is not necessary for transforming the plants by other methods such as microporation, microinjection, electroporation, polyethylene glycol, etc.

In general, plant transformation methods involve transferring heterologous DNA into target plant cells (e.g. immature or mature embryos, suspension cultures, undifferentiated callus, protoplasts, etc.), followed by applying a maximum threshold 10 level of appropriate selection (depending on the selectable marker gene) to recover the transformed plant cells from a group of untransformed cell mass. Explants are typically transferred to a fresh supply of the same medium and cultured routinely. Subsequently, the transformed cells are differentiated into shoots after placing on regeneration medium supplemented with a maximum threshold level of selecting agent. The shoots are then 15 transferred to a selective rooting medium for recovering rooted shoot or plantlet. The transgenic plantlet then grows into a mature plant and produces fertile seeds (e.g. Hiei *et al.* (1994) *The Plant Journal* 6: 271-282; Ishida *et al.* (1996) *Nature Biotechnology* 14: 745-750). Explants are typically transferred to a fresh supply of the same medium and cultured routinely. A general description of the techniques and methods for generating 20 transgenic plantlets are found in Ayres and Park, 1994 (*Critical Reviews in Plant Science* 13: 219-239) and Bommireddi and Jauhar, 1997 (*Maydica* 42: 107-120). Since the transformed material contains many cells; both transformed and non-transformed cells are present in any piece of subjected target callus or tissue or group of cells. The ability to kill non-transformed cells and allow transformed cells to proliferate results in 25 transformed plant cultures. Often, the ability to remove non-transformed cells is a limitation to rapid recovery of transformed plant cells and successful generation of transgenic plants.

Generation of transgenic plants may be performed by one of several methods, including but not limited to introduction of heterologous DNA by *Agrobacterium* into 30 plant cells (*Agrobacterium*-mediated transformation), bombardment of plant cells with heterologous foreign DNA adhered to particles, and various other non-particle direct-

mediated methods (e.g. Hiei *et al.* (1994) *The Plant Journal* 6: 271-282; Ishida *et al.* (1996) *Nature Biotechnology* 14: 745-750; Ayres and Park (1994) *Critical Reviews in Plant Science* 13: 219-239; Bommneni and Jauhar (1997) *Maydica* 42: 107-120) to transfer DNA.

5 Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, i.e., monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs 10 *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (U.S. Patent No. 5,563,055; U.S. Patent No. 5,981,840), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, U.S. Patent No. 4,945,050; U.S. Patent No. 5,879,918; U.S. Patent No. 5,886,244; U.S. Patent No. 5,932,782; Tomes *et al.* (1995) "Direct DNA 15 Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); McCabe *et al.* (1988) *Biotechnology* 6:923-926); aerosol beam transformation (U.S. Published Application No. 20010026941; U.S. Patent No. 4,945,050; International Publication No. WO 91/00915; U.S. Published Application No. 2002015066); and Lec1 20 transformation (WO 00/28058). Also see Weissinger *et al.* (1988) *Ann. Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37; Christou *et al.* (1988) *Plant Physiol.* 87:671-674; McCabe *et al.* (1988) *Bio/Technology* 6:923-926; Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182; Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740; 25 Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309; U.S. Patent No. 5,240,855; U.S. Patent Nos. 5,322,783 and 5,324,646; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin); Klein *et al.* (1988) *Plant Physiol.* 91:440-444; Hooykaas-Van Slogteren *et al.* (1984) *Nature (London)* 311:763-764; U.S. Patent No. 5,736,369; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae); De Wet *et al.* (1985) in *The Experimental*

Manipulation of Ovule Tissues, ed. Chapman *et al.* (Longman, New York), pp. 197-209; Kaeppeler *et al.* (1990) *Plant Cell Reports* 9:415-418 and Kaeppeler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566; D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505; Li *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413; Osjoda *et al.* (1996) *Nature Biotechnology* 14:745-750; all of which are herein incorporated by reference.

Following integration of heterologous foreign DNA into plant cells, one then applies a maximum threshold level of appropriate selection in the medium to kill the untransformed cells and separate and proliferate the putatively transformed cells that 10 survive from this selection treatment by transferring regularly to a fresh medium. By continuous passage and challenge with appropriate selection, one identifies and proliferates the cells that are transformed with the plasmid vector. Then molecular and biochemical methods will be used for confirming the presence of the integrated heterologous gene of interest in the genome of transgenic plant.

15 The cells that have been transformed may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having constitutive expression of the desired phenotypic characteristic identified. Two or more generations may be grown to 20 ensure that expression of the desired phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure expression of the desired phenotypic characteristic has been achieved. In this manner, the present invention provides transformed seed (also referred to as "transgenic seed") having a nucleotide construct of the invention, for example, an expression cassette of the invention, stably incorporated 25 into their genome.

The delta-endotoxin or delta-endotoxin-associated sequences of the invention may be provided in expression cassettes for expression in the plant of interest. The cassette will include 5' and 3' regulatory sequences operably linked to a sequence of the invention. By "operably linked" is intended a functional linkage between a promoter and a second 30 sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that

the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes.

5 Such an expression cassette is provided with a plurality of restriction sites for insertion of the delta-endotoxin or delta-endotoxin-associated sequence to be under the transcriptional regulation of the regulatory regions.

The expression cassette will include in the 5'-3' direction of transcription, a transcriptional and translational initiation region (i.e., a promoter), a DNA sequence of 10 the invention, and a transcriptional and translational termination region (i.e., termination region) functional in plants. The promoter may be native or analogous, or foreign or heterologous, to the plant host and/or to the DNA sequence of the invention.

15 Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. Where the promoter is "native" or "homologous" to the plant host, it is intended that the promoter is found in the native plant into which the promoter is introduced. Where the promoter is "foreign" or "heterologous" to the DNA sequence of the invention, it is intended that the promoter is not the native or naturally occurring promoter for the operably linked DNA sequence of the invention.

The termination region may be native with the transcriptional initiation region, 20 may be native with the operably-linked DNA sequence of interest, may be native with the plant host, or may be derived from another source (i.e., foreign or heterologous to the promoter, the DNA sequence of interest, the plant host, or any combination thereof).

Convenient termination regions are available from the Ti-plasmid of *A. tumefaciens*, such 25 as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; and Joshi *et al.* (1987) *Nucleic Acid Res.* 15:9627-9639.

30 Where appropriate, the gene(s) may be optimized for increased expression in the transformed host cell. That is, the genes can be synthesized using host cell-preferred codons for improved expression, or may be synthesized using codons at a host-preferred

codon usage frequency. Generally, the GC content of the gene will be increased. See, for example, Campbell and Gowri (1990) *Plant Physiol.* 92:1-11 for a discussion of host-preferred codon usage. Methods are known in the art for synthesizing plant-preferred genes. See, for example, U.S. Patent Nos. 6,320,100; 6,075,185; 5,380,831; and 5 5,436,391, U.S. Published Application Nos. 20040005600 and 20010003849, and Murray *et al.* (1989) *Nucleic Acids Res.* 17:477-498, herein incorporated by reference.

In one embodiment, the nucleic acids of interest are targeted to the chloroplast for expression. In this manner, where the nucleic acid of interest is not directly inserted into the chloroplast, the expression cassette will additionally contain a nucleic acid encoding a 10 transit peptide to direct the gene product of interest to the chloroplasts. Such transit peptides are known in the art. See, for example, Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9:104-126; Clark *et al.* (1989) *J. Biol. Chem.* 264:17544-17550; Della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res. Commun.* 196:1414-1421; and Shah *et al.* (1986) *Science* 233:478-481.

15 Methods for transformation of chloroplasts are known in the art. See, for example, Svab *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:8526-8530; Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA* 90:913-917; Svab and Maliga (1993) *EMBO J.* 12:601-606. The method relies on particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous 20 recombination. Additionally, plastid transformation can be accomplished by transactivation of a silent plastid-borne transgene by tissue-preferred expression of a nuclear-encoded and plastid-directed RNA polymerase. Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:7301-7305.

The nucleic acids of interest to be targeted to the chloroplast may be optimized for 25 expression in the chloroplast to account for differences in codon usage between the plant nucleus and this organelle. In this manner, the nucleic acids of interest may be synthesized using chloroplast-preferred codons. See, for example, U.S. Patent No. 5,380,831, herein incorporated by reference.

Evaluation of Plant Transformation

Following introduction of heterologous foreign DNA into plant cells, the transformation or integration of heterologous gene in the plant genome is confirmed by various methods such as analysis of nucleic acids, proteins and metabolites associated 5 with the integrated gene.

PCR Analysis: PCR analysis is a rapid method to screen transformed cells, tissue or shoots for the presence of incorporated gene at the earlier stage before transplanting into the soil (Sambrook and Russell, 2001). PCR is carried out using oligonucleotide primers 10 specific to the gene of interest or *Agrobacterium* vector background, etc.

Southern Analysis: Plant transformation is confirmed by Southern blot analysis of genomic DNA (Sambrook and Russell, 2001). In general, total DNA is extracted from the transformant, digested with appropriate restriction enzymes, fractionated in an 15 agarose gel and transferred to a nitrocellulose or nylon membrane. The membrane or “blot” then is probed with, for example, radiolabeled ^{32}P target DNA fragment to confirm the integration of introduced gene in the plant genome according to standard techniques (Sambrook and Russell, 2001. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

20 **Northern Analysis:** RNA is isolated from specific tissues of transformant, fractionated in a formaldehyde agarose gel, blotted onto a nylon filter according to standard procedures that are routinely used in the art (Sambrook, J., and Russell, D.W. 2001. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). Expression of RNA encoded by the delta-endotoxin or delta-endotoxin-associated is then tested by hybridizing the filter to a radioactive probe derived from a 25 delta-endotoxin or delta-endotoxin-associated protein, by methods known in the art (Sambrook and Russell, 2001).

30 **Western blot and Biochemical assays:** Western blot and biochemical assays and the like may be carried out on the transgenic plants to confirm the presence of protein encoded by

the delta-endotoxin or delta-endotoxin-associated gene by standard procedures (Sambrook, J., and Russell, D.W. 2001. *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY) using antibodies that bind to one or more epitopes present on the delta-endotoxin or delta-endotoxin-associated 5 protein.

Pesticidal Activity in Plants

In another aspect of the invention, one may generate transgenic plants expressing delta-endotoxin or delta-endotoxin-associated proteins that have pesticidal activity.

10 Methods described above by way of example may be utilized to generate transgenic plants, but the manner in which the transgenic plant cells are generated is not critical to this invention. Methods known or described in the art such as *Agrobacterium*-mediated transformation, aerosol beam, biolistic transformation, and non-particle-mediated methods may be used at the discretion of the experimenter. Plants expressing delta- 15 endotoxin or delta-endotoxin-associated proteins may be isolated by common methods described in the art, for example by transformation of callus, selection of transformed callus, and regeneration of fertile plants from such transgenic callus. In such process, one may use any gene as a selectable marker so long as its expression in plant cells confers ability to identify or select for transformed cells.

20 A number of markers have been developed for use with plant cells, such as resistance to chloramphenicol, the aminoglycoside G418, hygromycin, or the like. Other genes that encode a product involved in chloroplast metabolism may also be used as selectable markers. For example, genes that provide resistance to plant herbicides such as glyphosate, bromoxynil, or imidazolinone may find particular use. Such genes have 25 been reported (Stalker *et al.* (1985) *J. Biol. Chem.* 263:6310-6314 (bromoxynil resistance nitrilase gene); and Sathasivan *et al.* (1990) *Nucl. Acids Res.* 18:2188 (AHAS imidazolinone resistance gene).

30 Fertile plants expressing a delta-endotoxin or a delta-endotoxin-associated protein may be tested for pesticidal activity, and the plants showing optimal activity selected for further breeding. Methods are available in the art to assay for pest activity. Generally, the

protein is mixed and used in feeding assays. See, for example Marrone *et al.* (1985) *J. of Economic Entomology* 78:290-293.

Use in Pesticidal Control

5 General methods for employing the strains of the invention in pesticide control or in engineering other organisms as pesticidal agents are known in the art. See, for example U.S. Patent No. 5,039,523 and EP 0480762A2.

The *Bacillus* strains of the invention or the microorganisms which have been genetically altered to contain the pesticidal gene and protein may be used for protecting 10 agricultural crops and products from pests. In one aspect of the invention, whole, i.e., unlysed, cells of a toxin (pesticide)-producing organism are treated with reagents that prolong the activity of the toxin produced in the cell when the cell is applied to the environment of target pest(s).

Alternatively, the pesticide is produced by introducing a heterologous gene into a 15 cellular host. Expression of the heterologous gene results, directly or indirectly, in the intracellular production and maintenance of the pesticide. In one aspect of this invention, these cells are then treated under conditions that prolong the activity of the toxin produced in the cell when the cell is applied to the environment of target pest(s). The resulting product retains the toxicity of the toxin. These naturally encapsulated pesticides 20 may then be formulated in accordance with conventional techniques for application to the environment hosting a target pest, e.g., soil, water, and foliage of plants. See, for example EPA 0192319, and the references cited therein. Alternatively, one may formulate the cells expressing the genes of this invention such as to allow application of the resulting material as a pesticide.

25 The active ingredients of the present invention are normally applied in the form of compositions and can be applied to the crop area or plant to be treated, simultaneously or in succession, with other compounds. These compounds can be fertilizers, weed killers, cryoprotectants, surfactants, detergents, pesticidal soaps, dormant oils, polymers, and/or time-release or biodegradable carrier formulations that permit long-term dosing of a 30 target area following a single application of the formulation. They can also be selective herbicides, chemical insecticides, virucides, microbicides, amoebicides, pesticides,

fungicides, bacteriocides, nematocides, mollusocides or mixtures of several of these preparations, if desired, together with further agriculturally acceptable carriers, surfactants or application-promoting adjuvants customarily employed in the art of formulation. Suitable carriers and adjuvants can be solid or liquid and correspond to the substances ordinarily employed in formulation technology, e.g. natural or regenerated mineral substances, solvents, dispersants, wetting agents, tackifiers, binders or fertilizers. Likewise the formulations may be prepared into edible "baits" or fashioned into pest "traps" to permit feeding or ingestion by a target pest of the pesticidal formulation.

Preferred methods of applying an active ingredient of the present invention or an agrochemical composition of the present invention which contains at least one of the pesticidal proteins produced by the bacterial strains of the present invention are leaf application, seed coating and soil application. The number of applications and the rate of application depend on the intensity of infestation by the corresponding pest.

The composition may be formulated as a powder, dust, pellet, granule, spray, emulsion, colloid, solution, or such like, and may be preparable by such conventional means as desiccation, lyophilization, homogenation, extraction, filtration, centrifugation, sedimentation, or concentration of a culture of cells comprising the polypeptide. In all such compositions that contain at least one such pesticidal polypeptide, the polypeptide may be present in a concentration of from about 1% to about 99% by weight.

Lepidopteran or coleopteran pests may be killed or reduced in numbers in a given area by the methods of the invention, or may be prophylactically applied to an environmental area to prevent infestation by a susceptible pest. Preferably the pest ingests, or is contacted with, a pesticidally-effective amount of the polypeptide. By "pesticidally-effective amount" is intended an amount of the pesticide that is able to bring about death to at least one pest, or to noticeably reduce pest growth, feeding, or normal physiological development. This amount will vary depending on such factors as, for example, the specific target pests to be controlled, the specific environment, location, plant, crop, or agricultural site to be treated, the environmental conditions, and the method, rate, concentration, stability, and quantity of application of the pesticidally-effective polypeptide composition. The formulations may also vary with respect to

climatic conditions, environmental considerations, and/or frequency of application and/or severity of pest infestation.

The pesticide compositions described may be made by formulating either the bacterial cell, crystal and/or spore suspension, or isolated protein component with the 5 desired agriculturally-acceptable carrier. The compositions may be formulated prior to administration in an appropriate means such as lyophilized, freeze-dried, desiccated, or in an aqueous carrier, medium or suitable diluent, such as saline or other buffer. The formulated compositions may be in the form of a dust or granular material, or a suspension in oil (vegetable or mineral), or water or oil/water emulsions, or as a wettable 10 powder, or in combination with any other carrier material suitable for agricultural application. Suitable agricultural carriers can be solid or liquid and are well known in the art. The term "agriculturally-acceptable carrier" covers all adjuvants, inert components, dispersants, surfactants, tackifiers, binders, etc. that are ordinarily used in pesticide formulation technology; these are well known to those skilled in pesticide formulation.

15 The formulations may be mixed with one or more solid or liquid adjuvants and prepared by various means, e.g., by homogeneously mixing, blending and/or grinding the pesticidal composition with suitable adjuvants using conventional formulation techniques. Suitable formulations and application methods are described in U.S. Patent No. 6,468,523, herein incorporated by reference.

20 "Pest" includes but is not limited to, insects, fungi, bacteria, nematodes, mites, ticks, and the like. Insect pests include insects selected from the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Coleoptera, Lepidoptera, and Diptera.

25 Insect pests include insects selected from the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Coleoptera and Lepidoptera. Insect pests of the invention for the major crops include: Maize: *Ostrinia nubilalis*, European corn borer; *Agrotis ipsilon*, black cutworm; 30 *Helicoverpa zea*, corn earworm; *Spodoptera frugiperda*, fall armyworm; *Diatraea grandiosella*, southwestern corn borer; *Elasmopalpus lignosellus*, lesser cornstalk borer;

Diatraea saccharalis, sugarcane borer; *Diabrotica virgifera*, western corn rootworm; *Diabrotica longicornis barberi*, northern corn rootworm; *Diabrotica undecimpunctata howardi*, southern corn rootworm; *Melanotus* spp., wireworms; *Cyclocephala borealis*, northern masked chafer (white grub); *Cyclocephala immaculata*, southern masked chafer (white grub); *Popillia japonica*, Japanese beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*, corn leaf aphid; *Anuraphis maidiradicis*, corn root aphid; *Blissus leucopterus leucopterus*, chinch bug; *Melanoplus femur-rubrum*, redlegged grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Hylemya platura*, seedcorn maggot; *Agromyza parvicornis*, corn blot leafminer; *Anaphothrips obscurus*, grass thrips; *Solenopsis milesta*, thief ant; *Tetranychus urticae*, twospotted spider mite; Sorghum: *Chilo partellus*, sorghum borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Feltia subterranea*, granulate cutworm; *Phyllophaga crinita*, white grub; *Eleodes*, *Conoderus*, and *Aeolus* spp., wireworms; *Oulema melanopus*, cereal leaf beetle; *Chaetocnema pulicaria*, corn flea beetle; *Sphenophorus maidis*, maize billbug; *Rhopalosiphum maidis*, corn leaf aphid; *Sipha flava*, yellow sugarcane aphid; *Blissus leucopterus leucopterus*, chinch bug; *Contarinia sorghicola*, sorghum midge; *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Wheat: *Pseudaletia unipunctata*, army worm; *Spodoptera frugiperda*, fall armyworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Agrotis orthogonia*, western cutworm; *Elasmopalpus lignosellus*, lesser cornstalk borer; *Oulema melanopus*, cereal leaf beetle; *Hypera punctata*, clover leaf weevil; *Diabrotica undecimpunctata howardi*, southern corn rootworm; Russian wheat aphid; *Schizaphis graminum*, greenbug; *Macrosiphum avenae*, English grain aphid; *Melanoplus femur-rubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Melanoplus sanguinipes*, migratory grasshopper; *Mayetiola destructor*, Hessian fly; *Sitodiplosis mosellana*, wheat midge; *Meromyza americana*, wheat stem maggot; *Hylemya coarctata*, wheat bulb fly; *Frankliniella fusca*, tobacco thrips; *Cephus cinctus*, wheat stem sawfly; *Aceria tulipae*, wheat curl mite; Sunflower: *Suleima helianthana*, sunflower bud moth; *Homoeosoma electellum*, sunflower moth; *zygogramma exclamacionis*, sunflower beetle; *Bothyrus gibbosus*, carrot beetle; *Neolasioptera*

murtfeldtiana, sunflower seed midge; Cotton: *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Spodoptera exigua*, beet armyworm; *Pectinophora gossypiella*, pink bollworm; *Anthonomus grandis*, boll weevil; *Aphis gossypii*, cotton aphid; *Pseudatomoscelis seriatus*, cotton fleahopper; *Trialeurodes abutilonea*,
5 bandedwinged whitefly; *Lygus lineolaris*, tarnished plant bug; *Melanoplus femur-rubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Thrips tabaci*, onion thrips; *Frankliniella fusca*, tobacco thrips; *Tetranychus cinnabarinus*, carmine spider mite; *Tetranychus urticae*, twospotted spider mite; Rice: *Diatraea saccharalis*, sugarcane borer; *Spodoptera frugiperda*, fall armyworm; *Helicoverpa zea*, corn earworm;
10 *Colaspis brunnea*, grape colaspis; *Lissorhoptrus oryzophilus*, rice water weevil; *Sitophilus oryzae*, rice weevil; *Nephrotettix nigropictus*, rice leafhopper; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; Soybean: *Pseudoplusia includens*, soybean looper; *Anticarsia gemmatalis*, velvetbean caterpillar; *Plathypena scabra*, green cloverworm; *Ostrinia nubilalis*, European corn borer; *Agrotis epsilon*, black cutworm; *Spodoptera exigua*, beet armyworm; *Heliothis virescens*, cotton budworm; *Helicoverpa zea*, cotton bollworm; *Epilachna varivestis*, Mexican bean beetle; *Myzus persicae*, green peach aphid; *Empoasca fabae*, potato leafhopper; *Acrosternum hilare*, green stink bug; *Melanoplus femur-rubrum*, redlegged grasshopper; *Melanoplus differentialis*, differential grasshopper; *Hylemya platura*, seedcorn maggot; *Sericothrips variabilis*, soybean thrips; *Thrips tabaci*, onion thrips; *Tetranychus turkestanus*, strawberry spider mite; *Tetranychus urticae*, twospotted spider mite; Barley: *Ostrinia nubilalis*, European corn borer; *Agrotis epsilon*, black cutworm; *Schizaphis graminum*, greenbug; *Blissus leucopterus leucopterus*, chinch bug; *Acrosternum hilare*, green stink bug; *Euschistus servus*, brown stink bug; *Delia platura*, seedcorn maggot; *Mayetiola destructor*, Hessian fly; *Petrobia latens*, brown wheat mite; Oil Seed Rape: *Brevicoryne brassicae*, cabbage aphid; *Phyllotreta cruciferae*, Flea beetle; *Mamestra configurata*, Bertha armyworm; *Plutella xylostella*, Diamond-back moth; *Delia* spp., Root maggots.

Nematodes include parasitic nematodes such as root-knot, cyst, and lesion nematodes, including *Heterodera* spp., *Meloidogyne* spp., and *Globodera* spp.; particularly members of the cyst nematodes, including, but not limited to, *Heterodera glycines* (soybean cyst nematode); *Heterodera schachtii* (beet cyst nematode);

Heterodera avenae (cereal cyst nematode); and *Globodera rostochiensis* and *Globodera pallida* (potato cyst nematodes). Lesion nematodes include *Pratylenchus* spp.

The following examples are offered by way of illustration and not by way of limitation.

5

EXPERIMENTAL

Example 1. Extraction of Plasmid DNA

A pure culture of strain ATX13026 was grown in large quantities of rich media. The culture was spun to harvest the cell pellet. The cell pellet was then prepared by 10 treatment with SDS by methods known in the art, resulting in breakage of the cell wall and release of DNA. Proteins and large genomic DNA was then precipitated by a high salt concentration. The plasmid DNA was then precipitated by standard ethanol precipitation. The plasmid DNA was separated from any remaining chromosomal DNA by high-speed centrifugation through a cesium chloride gradient. The DNA was 15 visualized in the gradient by UV light and the band of lower density (i.e. the lower band) was extracted using a syringe. This band contained the plasmid DNA from strain ATX 13026. The quality of the DNA was checked by visualization on an agarose gel by methods known in the art.

20 Example 2. Cloning of Genes

The purified plasmid DNA was sheared into 5-10 kb sized fragments and the 5' and 3' single stranded overhangs repaired using T4 DNA polymerase and Klenow fragment in the presence of all four dNTPs, as known in the art. Phosphates were then attached to the 5' ends by treatment with T4 polynucleotide kinase, as known in the art. 25 The repaired DNA fragments were then ligated overnight into a standard high copy vector (i.e. pBluescript SK+), suitably prepared to accept the inserts as known in the art (for example by digestion with a restriction enzyme producing blunt ends).

The quality of the library was analyzed by digesting a subset of clones with a restriction enzyme known to have a cleavage site flanking the cloning site. A high 30 percentage of clones were determined to contain inserts, with an average insert size of 5-6 kb.

Example 3. High Throughput Sequencing of Library Plates

Once the shotgun library quality was checked and confirmed, colonies were grown in a rich broth in 2 ml 96-well blocks overnight at 37°C at a shaking speed of 350 rpm. The blocks were spun to harvest the cells to the bottom of the block. The blocks 5 were then prepared by standard alkaline lysis prep in a high throughput format.

The end sequences of clones from this library were then determined for a large number of clones from each block in the following way: The DNA sequence of each clone chosen for analysis was determined using the fluorescent dye terminator sequencing technique (Applied Biosystems) and standard primers flanking each side of 10 the cloning site. Once the reactions had been carried out in the thermocycler, the DNA was precipitated using standard ethanol precipitation. The DNA was resuspended in water and loaded onto a capillary sequencing machine. Each library plate of DNA was sequenced from either end of the cloning site, yielding two reads per plate over each insert.

15

Example 4. Assembly and Screening of Sequencing Data

DNA sequences obtained were compiled into an assembly project and aligned together to form contigs. This can be done efficiently using a computer program, such as Vector NTi, or alternatively by using the Pred/Phrap suite of DNA alignment and 20 analysis programs. These contigs, along with any individual read that may not have been added to a contig, were compared to a compiled database of all classes of known pesticidal genes. Contigs or individual reads identified as having identity to a known endotoxin or pesticidal gene were analyzed further. Among the sequences obtained, clone pAX008 contained DNA identified as having homology to known endotoxin genes.

25 Therefore, pAX008 was selected for further sequencing.

Example 5. Sequencing of pAX008, and Identification of AXMI-008

Primers were designed to anneal to pAX008, in a manner such that DNA sequences generated from such primers will overlap existing DNA sequence of the 30 clone(s). This process, known as “oligo walking,” is well known in the art. This process was utilized to determine the entire DNA sequence of the region exhibiting homology to

a known endotoxin gene. In the case of pAX008, this process was used to determine the DNA sequence of the entire clone, resulting in a single nucleotide sequence. The completed DNA sequence was then placed back into the original large assembly for further validation. This allowed incorporation of more DNA sequence reads into the 5 contig, resulting in multiple reads of coverage over the entire region.

Analysis of the DNA sequence of pAX008 by methods known in the art identified an open reading frame with homology to known delta endotoxin genes. This open reading frame is designated as AXMI-008. The DNA sequence of AXMI-008 is provided as SEQ ID NO:1, and the amino acid sequence of the predicted AXMI-008 protein is provided in 10 SEQ ID NO:3. An alternate start site for AXMI-008 at nucleotide 177 of SEQ ID NO:1 generates the amino acid sequence provided as SEQ ID NO:5. Further analysis identified an open reading frame immediately 3' to the end of the AXMI-008 open reading frame. This predicted amino acid sequence of this orf, referred to herein as AXMI-008orf2, is provided in SEQ ID NO:7.

15

Example 6. Homology of AXMI-008 to Known Endotoxin Genes

Searches of DNA and protein databases with the DNA sequence and amino acid sequence of AXMI-008 reveal that AXMI-008 is homologous to known endotoxins.

Blast searches identify *cry40Aa* as having the strongest block of homology, and 20 alignment of AXMI-008 protein (SEQ ID NO:3) to a large set of endotoxin proteins shows that the most homologous proteins is *cry40Aa*. The overall amino acid identity of *cry40Aa* to AXMI-008 is 66% (see Table 1). Inspection of the amino acid sequence of AXMI-008 suggests that it does not contain a C-terminal non-toxic domain as is present 25 in several endotoxin families. By removing this C-terminal protein of the toxins from the alignment, the alignment reflects the amino acid identify present solely in the toxin domains (see Table 1, column three). This 'trimmed' alignment is shown in Figure 1.

30

Table 1. Amino Acid Identity of AXMI-008 with Exemplary Endotoxin Classes

Endotoxin	Percent Amino Acid Identity to AXMI-008	Percent Amino Acid Identity of truncated Toxins to AXMI-008
<i>cry1Aa</i>	11%	20%
<i>cry1Ac</i>	11%	20%
<i>cry1Ia</i>	22%	22%
<i>cry2A</i>	10%	10%
<i>cry3Aa</i>	21%	21%
<i>cry3Bb</i>	21%	21%
<i>cry4Aa</i>	13%	21%
<i>cry4Ba</i>	13%	20%
<i>cry6Aa</i>	5%	5%
<i>cry7Aa</i>	12%	20%
<i>cry8Aa</i>	13%	22%
<i>cry10Aa</i>	20%	20%
<i>cry16Aa</i>	22%	22%
<i>cry19Ba</i>	21%	22%
<i>cry24Aa</i>	26%	26%
<i>cry25Aa</i>	23%	23%
<i>cry39Aa</i>	25%	25%
<i>cry40Aa</i>	66%	66%

Example 7. The ORF Immediately Downstream of AXMI-008 is Homologous to Downstream ORFs of Several Endotoxins

5 The open reading frame immediately downstream (3') to the AXMI-008 coding region has homology to known endotoxin-related proteins. Blast searches identify *crybun3orf2* (the downstream orf of *cry40Aa*) as having the strongest block of homology. Several other orf-2 like proteins are present in databases, and an alignment of AXMI-008 protein (SEQ ID NO:3) to a set of these proteins is shown in Figure 2. These proteins 10 also share homology to the C-terminal non-toxic domain of *cry4Aa* and *cry4Ba*. The overall amino acid identity of AXMI-8-orf2 to *cry40Aaorf2* is 86% (see Table 2).

Table 2. Amino acid identity of AXMI-008-orf2 to related proteins

Protein	Percent amino acid identity to AXMI-008-orf2
<i>crybun3orf2 (cry40Aa orf2)</i>	86%
<i>crybun2orf2 (cry39Aa orf2)</i>	85%
<i>cry19Aorf2</i>	62%
C-terminus <i>cry4Aa</i>	53%
C-terminus <i>cry4Ba</i>	54%

Example 8. Assays for Pesticidal Activity

5 The ability of a pesticidal protein to act as a pesticide upon a pest is often assessed in a number of ways. One way well known in the art is to perform a feeding assay. In such a feeding assay, one exposes the pest to a sample containing either compounds to be tested, or control samples. Often this is performed by placing the material to be tested, or a suitable dilution of such material, onto a material that the pest will ingest, such as an

10 artificial diet. The material to be tested may be composed of a liquid, solid, or slurry. The material to be tested may be placed upon the surface and then allowed to dry.

Alternatively, the material to be tested may be mixed with a molten artificial diet, then dispensed into the assay chamber. The assay chamber may be, for example, a cup, a dish, or a well of a microtiter plate.

15 Assays for sucking pests (for example aphids) may involve separating the test material from the insect by a partition, ideally a portion that can be pierced by the sucking mouthparts of the sucking insect, to allow ingestion of the test material. Often the test material is mixed with a feeding stimulant, such as sucrose, to promote ingestion of the test compound.

20 Other types of assays can include microinjection of the test material into the mouth, or gut of the pest, as well as development of transgenic plants, followed by test of the ability of the pest to feed upon the transgenic plant. Plant testing may involve isolation of the plant parts normally consumed, for example, small cages attached to a leaf, or isolation of entire plants in cages containing insects.

Other methods and approaches to assay pests are known in the art, and can be found, for example in Robertson, J. L. & H. K. Preisler. 1992. *Pesticide bioassays with arthropods*. CRC, Boca Raton, FL. Alternatively, assays are commonly described in the journals "Arthropod Management Tests" and "Journal of Economic Entomology" or 5 by discussion with members of the Entomological Society of America (ESA).

Example 9. Bioassay of pAX008 on *Trichoplusia ni* (Cabbage Looper)

An *Escherichia coli* strain containing pAX008, as well as a culture of untransformed *Escherichia coli* were grown in 2 ml of LB Broth (Luria-Bertani Broth, 10 Becton Dickinson & Company, Sparks, Md.) for 24 hours at 37° C with agitation at 250 rpm. pAX-008 was grown in LB containing the appropriate antibiotic to select for maintenance of the plasmid in *E. coli*.

Bioassays were performed using artificial diet (Multiple Species Diet, Southland Products, Lake Village, Arkansas) in 24 well tissue culture plates. Bioassays were carried 15 out by applying the *Escherichia coli* culture containing pAX-008 to the diet surface and allowing the diet surface to dry. The strains were applied as whole cultures to the diet at a concentration of 40 µl of culture per well. The bioassays were held in the dark at 25° C and 65% relative humidity. Trays were sealed with Breathe Easy Sealing Tape (Diversified Biotech, Boston, MA). Results were recorded at 5 days.

20

Table 3. Assay of AXMI-008 clone pAX008 on *T. ni*

Sample	# Dead/ Total	% Mortality
pAX-008	6/6	100%
Negative Control	0/13	0%

Example 10. Expression of AXMI-008 in *Bacillus*

The 1,890 base pair insecticidal *axmi008* gene was amplified by PCR from 25 pAX008, and cloned into the *Bacillus* Expression vector pAX922 by methods well known in the art. The resulting clone, pAX922, expressed AXMI-008 protein when transformed into cells of a *cry(-)* *Bacillus thuringiensis* strain. The *Bacillus* strain containing pAX922 and expressing the AXMI-008 insecticidal protein may be cultured

on a variety of conventional growth media. A *Bacillus* strain containing pAX922 was grown in CYS media (10 g/l Bacto-casitone; 3 g/l yeast extract; 6 g/l KH₂PO₄; 14 g/l K₂HPO₄; 0.5 mM MgSO₄; 0.05 mM MnCl₂; 0.05 mM FeSO₄), until sporulation was evident by microscopic examination. Samples were prepared, and AXMI-008 was tested
5 for insecticidal activity in bioassays against important insect pests.

Example 11. Bioassay of AXMI-008 on *Tenebrio molitor*

Samples of *Bacillus* cultures expressing AXMI-008 were prepared and tested for
pesticidal activity on *Tenebrio molitor*. When pAX 922 is prepared as an insoluble
10 fraction at pH 4.0 it showed activity against commonly called the yellow mealworm.

Samples of AXMI-008 were prepared from a culture of a *Bacillus* strain
containing pAX 922. The bioassay sample was prepared by growing a culture in CYS
media for 4 days, until sporulation. The sample was centrifuged at 10,000 rpm for 10
minutes and the supernatant discarded. The pellet was washed in 20mM Tris pH 8 and
15 spun at 10,000 rpm for 10 minutes. The supernatant was discarded and the pellet
resuspended in 3 mls of 50 mM Sodium Citrate and 25 mM Sodium Chloride with 2mM
DTT at pH 4. The sample was incubated at 37° C for 1 hour. After incubation the sample
was spun at 13,000 rpm for 10 minutes and the supernatant discarded. The pellet was
resuspended in 50 mM Sodium Citrate and 25 mM Sodium Chloride at pH 4.

20 Bioassays of samples on *Tenebrio molitor* were performed on an artificial diet
(Southern Corn Rootworm Diet, Bioserv, Frenchtown, NJ, #F9757B) in 24 well tissue
culture plates. The sample was applied as a surface treatment with a concentration of
Axmi008 at 8 ug/cm² and allowed to air dry. The insects were applied using a fine tip
brush. Bioassay trays were sealed with Breathe Easy Sealing Tape (Diversified Biotech,
25 Boston, MA) and incubated without light at 65% relative humidity, 25° C. for seven
days and results recorded.

Table 4. Assay of AXMI-008 clone pAX008 on *T. molitor*

Sample	# Dead/ Total	% Mortality
pAX922	3 of 4	75%*

* Remaining *Tenebrio molitor* was stunted. Stunting is observed as reduced larval size and growth, and severely reduced or minimal feeding. The insect may also demonstrate avoidance of the treated diet compared to the untreated diet.

5

Methods

To prepare CYS media: 10 g/l Bacto-casitone; 3 g/l yeast extract; 6 g/l KH₂PO₄; 14 g/l K₂HPO₄; 0.5 mM MgSO₄; 0.05 mM MnCl₂; 0.05 mM FeSO₄. The CYS mix should be pH 7, if adjustment is necessary. NaOH or HCl are preferred. The media is then autoclaved and 100 ml of 10X filtered glucose is added after autoclaving. If the resultant solution is cloudy it can be stirred at room temperature to clear.

Example 12. Vectoring of AXMI-008 for Plant Expression

The AXMI-008 coding region DNA is operably connected with appropriate promoter and terminator sequences for expression in plants. Such sequences are well known in the art and may include the rice actin promoter or maize ubiquitin promoter for expression in monocots, the *Arabidopsis* UBQ3 promoter or CaMV 35S promoter for expression in dicots, and the nos or PinII terminators. Techniques for producing and confirming promoter – gene – terminator constructs also are well known in the art.

20 The plant expression cassettes described above are combined with an appropriate plant selectable marker to aid in the selections of transformed cells and tissues, and ligated into plant transformation vectors. These may include binary vectors from *Agrobacterium*-mediated transformation or simple plasmid vectors for aerosol or biolistic transformation.

25

Example 13. Transformation of Maize Cells with AXMI-008

Maize ears are collected 8-12 days after pollination. Embryos are isolated from the ears, and those embryos 0.8-1.5 mm in size are used for transformation. Embryos are plated scutellum side-up on a suitable incubation media, such as DN62A5S media (3.98

g/L N6 Salts; 1 mL/L (of 1000x Stock) N6 Vitamins; 800 mg/L L-Asparagine; 100 mg/L Myo-inositol; 1.4 g/L L-Proline; 100 mg/L Casaminoacids; 50 g/L sucrose; 1 mL/L (of 1 mg/mL Stock) 2,4-D), and incubated overnight at 25°C in the dark.

5 The resulting explants are transferred to mesh squares (30-40 per plate), transferred onto osmotic media for 30-45 minutes, then transferred to a beaming plate (see, for example, PCT Publication No. WO/0138514 and U.S. Patent No. 5,240,842).

DNA constructs designed to express AXMI-008 in plant cells are accelerated into plant tissue using an aerosol beam accelerator, using conditions essentially as described in PCT Publication No. WO/0138514. After beaming, embryos are incubated for 30 min 10 on osmotic media, then placed onto incubation media overnight at 25°C in the dark. To avoid unduly damaging beamed explants, they are incubated for at least 24 hours prior to transfer to recovery media. Embryos are then spread onto recovery period media, for 5 days, 25°C in the dark, then transferred to a selection media. Explants are incubated in selection media for up to eight weeks, depending on the nature and characteristics of the 15 particular selection utilized. After the selection period, the resulting callus is transferred to embryo maturation media, until the formation of mature somatic embryos is observed. The resulting mature somatic embryos are then placed under low light, and the process of regeneration is initiated by methods known in the art. The resulting shoots are allowed to root on rooting media, and the resulting plants are transferred to nursery pots and 20 propagated as transgenic plants.

Materials

DN62A5S Media

Components	per liter	Source
Chu'S N6 Basal Salt Mixture (Prod. No. C 416)	3.98 g/L	Phytotechnology Labs
Chu's N6 Vitamin Solution (Prod. No. C 149)	1 mL/L (of 1000x Stock)	Phytotechnology Labs
L-Asparagine	800 mg/L	Phytotechnology Labs
Myo-inositol	100 mg/L	Sigma
L-Proline	1.4 g/L	Phytotechnology Labs
Casaminoacids	100 mg/L	Fisher Scientific
Sucrose	50 g/L	Phytotechnology Labs
2,4-D (Prod. No. D-7299)	1 mL/L (of 1 mg/mL Stock)	Sigma

15 Adjust the pH of the solution to pH to 5.8 with 1N KOH/1N KCl, add Gelrite (Sigma) to 3g/L, and autoclave. After cooling to 50°C, add 2 ml/L of a 5 mg/ml stock solution of Silver Nitrate (Phytotechnology Labs). Recipe yields about 20 plates.

Example 14. Transformation of AXMI-008 into Plant Cells by *Agrobacterium*-Mediated Transformation

Ears are collected 8-12 days after pollination. Embryos are isolated from the ears, and those embryos 0.8-1.5 mm in size are used for transformation. Embryos are plated 5 scutellum side-up on a suitable incubation media, and incubated overnight at 25°C in the dark. However, it is not necessary *per se* to incubate the embryos overnight. Embryos are contacted with an *Agrobacterium* strain containing the appropriate vectors for Ti plasmid mediated transfer for 5-10 min, and then plated onto co-cultivation media for 3 days (25°C in the dark). After co-cultivation, explants are transferred to recovery period media 10 for five days (at 25°C in the dark). Explants are incubated in selection media for up to eight weeks, depending on the nature and characteristics of the particular selection utilized. After the selection period, the resulting callus is transferred to embryo 15 maturation media, until the formation of mature somatic embryos is observed. The resulting mature somatic embryos are then placed under low light, and the process of regeneration is initiated as known in the art. The resulting shoots are allowed to root on rooting media, and the resulting plants are transferred to nursery pots and propagated as transgenic plants.

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. 20 All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that 25 certain changes and modifications may be practiced within the scope of the appended claims.